3030-Pos Board B722
Opening Windows into the Cell: Focused-Ion-Beam Milling for Cryo-Electron Tomography
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Cryo-electron tomography (cryo-ET) is a biophysical tool that provides unprecedented insights into the 3-D organization of cells in their native state at molecular resolution, free of artefacts such as fixation, staining, or labelling. To date, the main limitation of cryo-ET is the thickness of most cells, rendering them inaccessible to intermediate-voltage TEM. I will describe how we have adapted focused ion beam (FIB) milling to prepare 200-500 nm lamellae from intact cells, effectively opening large windows into the cell's interior at molecular resolution. Through image processing, macromolecular complexes can be localized and identified, enabling quantitative analysis of the organization and structure of molecular complexes in situ. Cryo-ET and FIB milling were used to study the nuclear pore complex, one of the largest macromolecular machines in the cell that selectively controls all traffic between the nucleus and the cytoplasm. Due to its sheer size, its local environment and its dynamic nature, determining its structure at molecular resolution remains a challenge for conventional techniques, and studies of nuclear transport lack a molecular scaffold onto which models can be interpreted. Combining FIB milling, cryo-ET, and image processing enable the study of the NPC in its native environment, performing its function, and free of the distortions caused by purification. This approach has produced the yeast NPC architecture at unparalleled resolution and revealed the structural dynamics of the NPC in action. Other uses of cryo-FIB/ET to study diverse cellular environments at molecular detail will be presented, including actin networks and the distribution of macromolecular complexes within organelles such as mitochondria. Finally, I will discuss new emerging technologies in EM, including direct detector devices and phase plates, and their impact on cryo-ET.

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CRYO-EM Atomic Model of Brome Mosaic Virus Derived from Direct Electron Detection Images and a Real-Space Model Optimization Protocol
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Recent advances in cryo-EM have enabled structure determination of macromolecules at near-atomic resolution. However, de novo structure determination remains susceptible to model bias and overfitting, potentially yielding an inaccurate structure even if resolution appears high. Here, we describe a complete workflow for data acquisition, processing, modeling, and validation of an RNA virus, Brome Mosaic Virus (BMV). Data was collected using "movies" with a direct electron detector in integrating mode with a cumulative exposure beyond the traditional radiation damage limit. Reconstruction based on randomly-generated initial models yielded a map at 3.3 and 3.8 Å resolution, with and without subunit averaging, respectively. We used this map to compute a de novo Cα backbone model, which was converted to an all-atom model and optimized with a newly-implemented real-space refinement protocol. The final de novo atomic model ranks in the top 99th percentile when compared to PDB structures at equivalent resolution using statistical scores routinely used in X-ray crystallography.

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Structural Visualization of Mitotic Cycle by Three-Dimensional Focused Ion Beam-Scanning Electron Microscope (FIB-SEM) with Nanoscale Resolution at Whole Cell Level
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To understand the frontier Biosciences, it is also important to observe the three-dimensional structure of biological molecules in cells and tissue. We describe a new biological application of FIB-SEM, which is normally used to visualize metals and ceramics surface, for the 3D reconstruction of an entire cell at a nanoscale resolution that lies between those of EM tomography and X-ray tomography.
We used FIB-SEM to visualize the 3D architecture of *Cyanidioschyzon merolae (C. marolae)* , which is thinking as the primitive unicellular red algae. *C. marolae* is the only eukaryotic organism which can control its chloroplast, mitochondria and cell division by light/dark adjustment. Because cell division is expected a basis of life, we can know the basic mechanism of eukaryotic cells by examining the structure of *C. merolae* in each division process. Our system could image simple individual double-membrane organelles like nucleus, chloroplast, and mitochondria, and single-membrane organelles like the ER, Golgi, lysosome and peroxisome inside *C. merolae* of 2-5 μm in length. We stained *C. merolae* cells with Hoechst 33342 and visualized them by fluorescence microscopy, succeeding to resolve the DNA of the chloroplast, mitochondria and cell nucleus. The position of the chloroplast was determined by the autofluorescence. Cells can be classified into five types (correspond by G1, S, G2 early, G2 late, M-phase) based on differences in shape, size and distribution of the organelles during mitosis. By effective synchronizing cells to a 6-h light/18-h dark cycle according to Moriyama T. *et al.* (*Microbiology* , **156** , 1730-7, 2010) with some modifications, we obtained > 70% S/M-phase cells. We will show you 3-D surface models of the different *C. merolae* cell types from the obtained sequential 2D-SEM images.

3033-Pos Board B725
Overcoming Patch-Potential Effects on the Surfaces of Tem Phase-Contrast Devices
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A Focused-Ion Beam (FIB) tool is used to fabricate thin-foil apertures that have an electron-opaque half-circle at the center, suspended by a beam extending from the outer edge. The opaque half-circle produces single-sideband contrast (also known as "schlieren", or Foucault knife-edge contrast) at low spatial frequencies, while still allowing one to use Scherzer defocus to provide optimal contrast transfer at intermediate and high spatial frequencies. While a requirement for such an aperture to be effective is that the electrostatic equipotential surface must conform closely to the physical surface of the device, this requirement is not well satisfied on the vacuum-side of any polycrystalline material, due to variation of the work function of difference crystal facets. We thus coat newly fabricated apertures with graphitic a-C to provide a surface with a uniform work function. We now report that heating such apertures to ~650 °C during use is effective in real-time "annealing" of the radiation damage that otherwise causes a local (patchy) change in phase shift to build up at the edge of the opaque half-circle. While this represents a significant advance, there remains an as-yet unsolved problem in achieving consistent fabrication of apertures that retain the desired uniformity of electrostatic potential when they are heated to ~650 °C. Current work is focused in two directions: (1) taking steps to enhance bonding of the a-C coating (i.e. prevent delamination), and (2) investigating other materials that may not need to be coated with a-C but that will (like a-C) be rad-hard, at least when heated to ~650 °C or less.

3034-Pos Board B726
Substrate Effects on Structural Studies using Cryo-Em
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In electron microscopy, glow discharge has been widely used to render the carbon film hydrophilic for better adhesion of aqueous solution. However, the target macromolecules (e.g. proteins) may also interact with the charge on the carbon film, which may introduce preferred orientation and/or some other artifacts. A promising alternative is to use two-dimensional (2D) crystal as a substrate. This method has been successfully employed to study DNA molecules, proteoliposomes, and multiprotein complexes. Here we will use liposomes (i.e. lipid vesicles) as a model system to study the effect of substrate on targeted structures. Four types of substrates will be studied: untreated carbon film, glow-discharged carbon film, holey carbon film, and 2D streptavidin crystal. Liposomes, doped with a few copies of biotinylated lipid and osmotically swollen to ensure a spherical shape, will be allowed to attach to the substrate before blotting and rapid freezing of the specimen. Then cryo-electron microscopy of tilted specimens and cryo-electron tomography will be employed, and the resulted images and tomographs will be compared with spherical liposome models. The deformation of the liposomes will be used to quantify the effect of the substrate on the targeted structure.

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A First Look into the 3D Structure of the TRPV2 Channel by Single-Particle Cryo-Em
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The TRPV2 channel is a ~385 kDa integral membrane protein complex that functions as a high-threshold thermosensor and is involved in calcium signaling